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MICROTUBULE PROTEIN

Identification in and Transport to Nerve Endings

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ABSTRACT

The subunit protein of microtubules, tubulin, has been demonstrated to be present in isolated nerve endings by gel electrophoresis, amino acid composition, and peptide mapping. The tubulin constitutes approximately 28% of the soluble protein of the nerve endings. The transport of tubulin to the nerve endings has been demonstrated and its relationship to slow transport is discussed.

INTRODUCTION

The protein subunit of microtubules, tubulin, is present in the brain in large quantity (1-3). Tubulin has a sedimentation velocity of 6S in the 120,000 dalton native dimeric form, binds colchicine (2-4), and is precipitable by vinblastine (5, 6, 7, 8). The role of microtubules in brain is undefined. As in other cells, they have been linked to morphogenesis and to stability of form. They also have been implicated in axoplasmic transport (8). Most studies of microtubular function in brain have relied on mitotic spindle inhibitors (19-21) as a tool for interruption of the integrity of the microtubule and the subsequent assay of the effect of this disruption. A serious difficulty in the interpretation of such experiments is a lack of knowledge of the transport and destination of tubulin itself. Previous studies have suggested (17) that tubulin is transported with the slow com-

ponent of transport, but this is based on indirect evidence. A second problem is that morphological studies (28) reveal few, if any, microtubules at nerve endings. Also, if tubulin is transported with slow flow, certain evidence suggests that slow flow may not enter the synaptic expansion itself (29).

To resolve these problems we have attempted to apply the axoplasmic flow technique of Barondes (11) in which flow is assessed by comparing the levels of labeled protein in isolated synaptosomes to levels of the same material in whole brain at varied time intervals after the injection of labeled precursor. The results demonstrate the transport of tubulin to the nerve ending and its presence in large quantity in the ending and lend support to the conclusion that this transport is in the slow component.

MATERIALS AND METHODS

Synaptosomal Isolation

CD 1 or BALB/c mice between the ages of 3 and 7 days were used for these experiments. Subcellular fractionation and isolation of the synaptosomal fraction were done by a slight modification of the procedure of Gray and Whittaker (10) as described previously (11). The major modification involves the washing of the crude mitochondrial fraction three times with 0.32 M sucrose to ensure removal of adsorbed soluble protein. The nerve ending (synaptosomal) fraction was lysed with 5 ml of water at 0°C and the lysed preparation was centrifuged at 100,000 *g* for 1 hr to yield a soluble component of the nerve ending fraction (NES) and a particulate component (NEP). In the experiments in which radioactive amino acid incorporation into brain protein was studied, mice received a single intracerebral injection of 10 μ Ci of L-leucine 4,5-³H (4 mCi/mole, New England Nuclear Corp., Boston, Mass.) in 10 μ l of isotonic saline in the temporal region of the brain 15 min, 90 min, or 24 hr before sacrifice. The whole soluble fraction (WS) was prepared by taking the supernatant from the crude mitochondrial preparation above and centrifuging it at 100,000 *g* for 1 hr and utilizing the supernatant for further study while discarding the pellet. The fraction provides an index of neuronal cell body levels of labeled materials for comparison with the levels in isolated synaptosomes. A major problem here is that the WS contains not only neural but also glial cytoplasm and quite possibly cytoplasm from nerve endings which may have ruptured during initial homogenization. The former problem is eased slightly by the fact that the immature mouse brain is relatively poor in glial cells.

Vinblastine Precipitation

The protein concentrations of the 100,000 *g* supernatant from whole brain homogenate (WS) and from the soluble component of the nerve ending fraction (NES) were adjusted to 1–2 mg/ml, and MgCl₂, sodium phosphate buffer (pH 7.0), and vinblastine sulfate were added to final concentrations of 0.01 M, 0.01 M, and 5×10^{-4} M, respectively. The vinblastine sulfate was obtained from Eli Lilly & Co., Indianapolis, Ind. The mixture was incubated at 37°C for 1/2 hr (5) and the precipitate was collected by centrifugation at 35,000 *g* for 15 min. Control experiments revealed 100% sedimentation of colchicine binding activity under these conditions, and polyacrylamide gels of supernatant and pellet revealed all protein which co-migrated with a tubulin standard to be precipitated. The purity of the precipitates was estimated at 90–95% by densitometric scans of gels stained in amido-Schwarz. Similar estimates of

purity were obtained by fractionation and counting of the gels after radioactive labeling of the protein. Since the results presented in this paper are based on liquid scintillation counting and protein determination on the entire precipitate, they will overestimate the actual amount of tubulin by 5–10% of the experimental figures. Therefore, all determinations of quantities of tubulin in the following sections are diminished by 10% from their experimental values to compensate for this error. Scans on such precipitates are shown in Fig. 2 *a*.

Since higher purity was desired for peptide mapping and amino acid composition studies, the vinblastine precipitates were resolubilized by dialysis against 0.01 M phosphate buffer, pH 7.0, and the final solution was clarified by centrifugation at 6000 *g* for 20 min. The final supernatant was then taken for study. The insoluble pellet amounted to 10–20% of the total protein of the original precipitate and contained up to 40% tubulin as determined by gel electrophoresis. The supernatant was 98–99% pure tubulin as determined by densitometry and counting of labeled protein. Typical gels of such a preparation are shown in Fig. 2 *b*.

Polyacrylamide Gel Electrophoresis

Protein samples were reduced in β -mercaptoethanol and alkylated with iodoacetamide in the presence of 8 M urea (12), except in the case of samples to be run on neutral sodium dodecyl sulfate (SDS) gels in which case the urea was omitted. Three types of gel systems were used: (a) the neutral pH gel system incorporating 0.1% sodium dodecyl sulfate (SDS) in which the proteins migrate according to molecular weight (13). The bands in this system tend to be quite broad; (b) the discontinuous gel system of Davis (14) modified by the inclusion of 8 M urea in the gel; (c) the Davis system as modified by Albert et al. (15) with the inclusion of both 8 M urea and 0.1% SDS in the gel and 0.1% SDS in the buffer; separation in this system is by molecular weight over the range of 30,000–135,000 daltons. The acrylamide concentration in each case was 7.5%. Gels were fixed overnight in a 50/50 mixture of 7% acetic acid in water and 7% acetic acid in methanol. Staining was done for 8 hr in amido-Schwarz, followed by electrophoretic destaining in a transverse destaining apparatus. Amido-Schwarz was chosen for staining because it represented a reasonable compromise between sensitivity and quantitation. The more sensitive Coomassie blue is nonlinear for tubulin, while amido-Schwarz gave better agreement with actual protein concentrations. Samples of WS and NES stained in amido-Schwarz and in the more quantitative fast green (30) differed by only a small percentage for any single peak.

Gels used for radioactive experiments were all run

with identical amounts of protein. The protein profiles within the NES and WS fractions remained constant with time as determined by densitometry. Differences exist, of course, between the profiles from NES and WS. Proteins were labeled with tritiated leucine, and a ^{14}C -labeled tubulin standard was used for calibration. Gel slices were solubilized for counting in NCS reagent and counted in Bray's solution (31).

Peptide Mapping

Protein samples were reduced and alkylated (12) and aliquots were checked for purity by gel electrophoresis. The samples were then dialyzed for 24 hr against 0.1 M ammonium carbonate at pH 8.5 followed by digestion with trypsin (DCC-treated, Calbiochem, Los Angeles, Calif.) at an enzyme-to-protein ratio of 1:50 for 14 hr at 37°C. Tubulin samples used were in the range of 300–400 μg for each map. Thin-layer peptide maps were prepared by the method of Ritschard (16). The tryptic peptides were spotted on 20-cm square silica gel G plates (Analtech, Inc., Wilmington, Del.) and chromatographed in an ascending propanol-acetic acid-water solvent system in a volume ratio of 7:1:2. The plates were dried in an oven at 100°C for 10 min and then electrophoresed in a flat-bed apparatus with paper wicks at 10,000 v for 90 min. The buffer for electro-

phoresis was pyridine-acetic acid-water in volume ratios of 1:10:489. The plates were dried at 110°C, and sprayed with ninhydrin in a trimethylpyridine-acetic acid buffer. The color was developed by heating the plates at 80°C for a few minutes.

Protein Determinations

Protein was determined colorimetrically by the method of Lowry et al. (32). The analysis was standardized using crystalline bovine serum albumin. Since the indole rings in vinblastine can interfere with the Lowry determination and give falsely high results, all samples were precipitated with 10% trichloroacetic acid (TCA) and washed twice in 10% TCA before analysis. No differences are seen between equal samples of tubulin, one of which was vinblastine precipitated and one not, after this treatment.

Amino Acid Analysis

Samples were hydrolyzed in 6 N HCl in sealed evacuated tubes at 110°C for 24 and 48 hr. The protein concentrations were made equal in all samples in order to control for possible side reactions between the amino acid and sugar moieties. Samples were analysed using a 4 hr, two-column methodology on a Jeolco 5AH analyser (Jeolco U. S. A. Inc., Medford, Mass.).

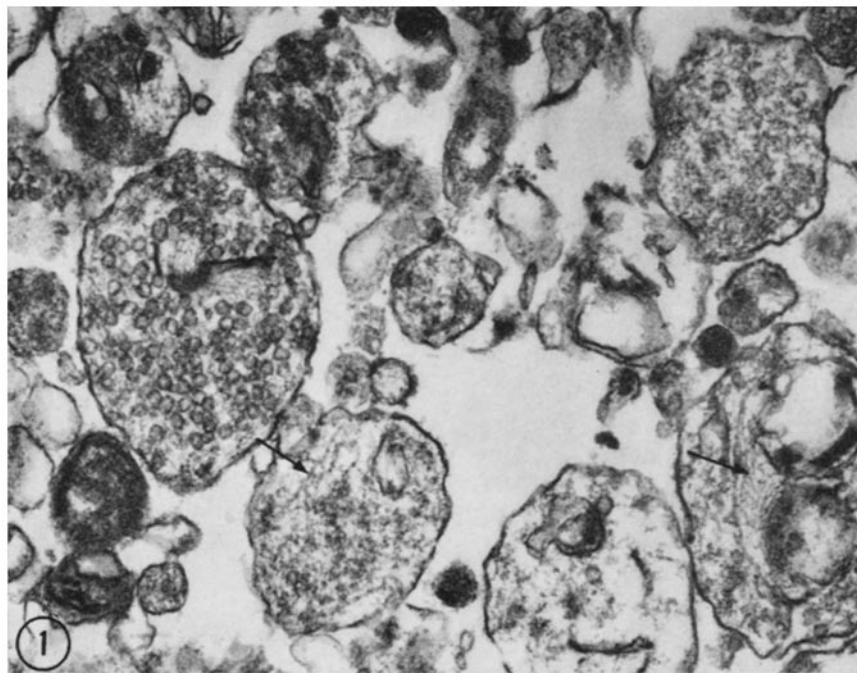


FIGURE 1 A typical field of isolated nerve endings prepared from 3–7 day old mice. Note presence of some neurotubules and neurofilaments (arrows). $\times 10,000$.

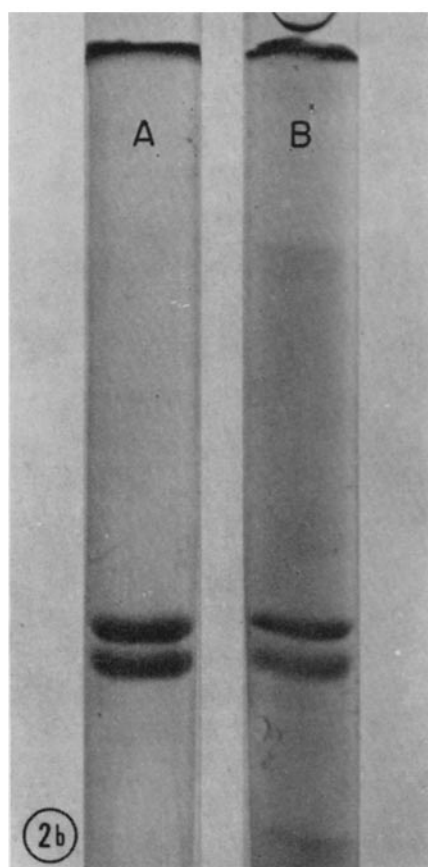
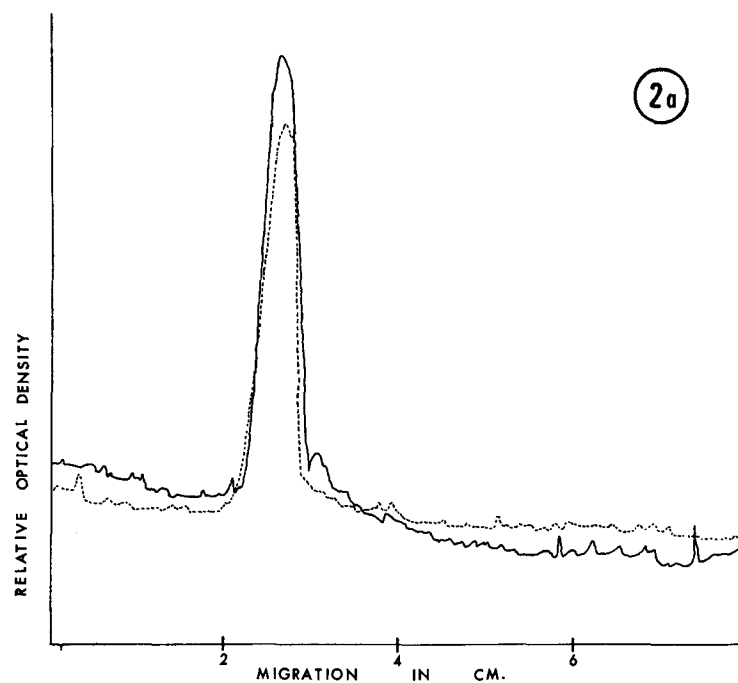


FIGURE 2 Fig. 2 *a*, Polyacrylamide gel electrophoresis (8 M urea gels; Materials and Methods, gel system *b*) of tubulin prepared from NES fraction (solid line) and WS fraction (dotted line). Gels were stained with amido-Schwarz and scanned on a Joyce-Loebl Chromoscan densitometer (Joyce, Loeb & Co., Inc., Burlington, Mass.). Migration is from left to right with anode at right. Fig. 2 *b*, photograph of polyacrylamide gels (8 M urea-SDS; Materials and Methods, gel system *c*) of tubulins prepared from NES fraction (*a*) and from WS fraction (*b*). The two bands in each are equal in concentration and may represent the monomers of the tubulin dimer or two species of tubulin.

TABLE I
*Amino Acid Compositions of Tubulin Purified from
NES and WS Fractions*

	WS Tubulin	NES Tubulin
Lysine	4.7	4.7
Histidine	2.4	2.4
Arginine	4.3	4.3
Aspartic acid	10.8	10.4
Threonine	6.6	6.6
Serine	6.7	6.7
Glutamic acid	13.7	13.7
Proline	4.5	4.7
Glycine	7.9	7.8
Alanine	8.1	8.4
1/2-Cystine	trace	trace
Valine	7.0	6.7
Methionine	2.8	2.7
Isoleucine	4.2	4.2
Leucine	9.3	9.3
Tyrosine	3.4	3.1
Phenylalanine	4.4	4.1
Tryptophan	not determined	

All values are expressed in moles per cent. Values for threonine and serine were corrected for destruction by extrapolation to zero time from 24 and 48 hr determinations.

Electron Microscopy

Pellets were fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer at pH 7.4 for 1 hr at room temperature and postfixed with 1% buffer osmium tetroxide for 1 hr. Dehydration was carried out in graded alcohols from 70% to absolute, followed by embedding in Epon 812. Sections were cut on a Reichart ultramicrotome (Reichart, Inc., Austria) and examined with a Siemens 1A electron microscope.

RESULTS

Identification of Microtubule Protein in the Soluble Components of the Nerve Ending Fraction

Isolated synaptosomes were obtained from the brains of immature mice at a high level of purity (Fig. 1). With the vinblastine precipitation procedure, 28% of the total protein of the NES fraction and 15% of the total protein of the WS fraction were recovered. Proteins from NES and WS purified by the vinblastine procedure co-migrated with microtubule protein purified from porcine brain by the method of Weisenberg et al. (3) on all

three gel systems which were used. Co-migration was established by examining the electrophoresis pattern of appropriate mixtures as well as with parallel gels. In the 8 M urea system, all samples ran as single discrete bands. In the 8 M urea-SDS system, all the samples ran as a well-resolved double band (Fig. 2 *b*) with apparent molecular weights of 56,000 and 53,000, respectively, on a calibrated gel. It is not clear whether such small differences in molecular weight are significant in this system. The splitting may represent resolution of two nonidentical tubulin monomers or the presence of two tubulins in brain (27). This splitting is obtained on single bands eluted from 8 M urea gels and rerun on this system. Each band seen can be eluted and rerun on the same system giving a single band.

Amino acid compositions of the proteins from WS and NES purified by the vinblastine procedure were virtually indistinguishable from each other (Table I). Comparative peptide maps of this material were also strikingly similar (Fig. 3). Of the 51 peptides detected, 43 appear to be the same in the two preparations.

The combination of results of the electrophoretic, amino acid, and peptide mapping studies established that the proteins purified by the vinblastine procedures from both NES and WS are similar and suggest that both of them are tubulin. The possibility that the tubulin found in the NES was due to contamination of the nerve ending fraction by adsorbed tubulin from WS was evaluated in the previous study (9). It was shown that, upon mixing radioactively labeled protein from WS with unlabeled homogenates and then isolating NES, less than 4% of the protein of NES represented contamination by WS. Even if all the WS contamination was tubulin, this would account for less than 25% of the tubulin recovered from NES. Homogenization of brain in the presence of labeled WS also revealed less than 4% contamination of NES by WS.

Transport of Tubulin to Nerve Endings

The specific activity of tubulin in NES and WS at a number of times after intracerebral injection of radioactive leucine was determined. The protein of WS was maximally labeled within 90 min after intracerebral injection of radioactive leucine, and the specific activity of this protein declined thereafter (Fig. 4 *a*). In contrast, the protein of NES was only slightly labeled at early time points, and the

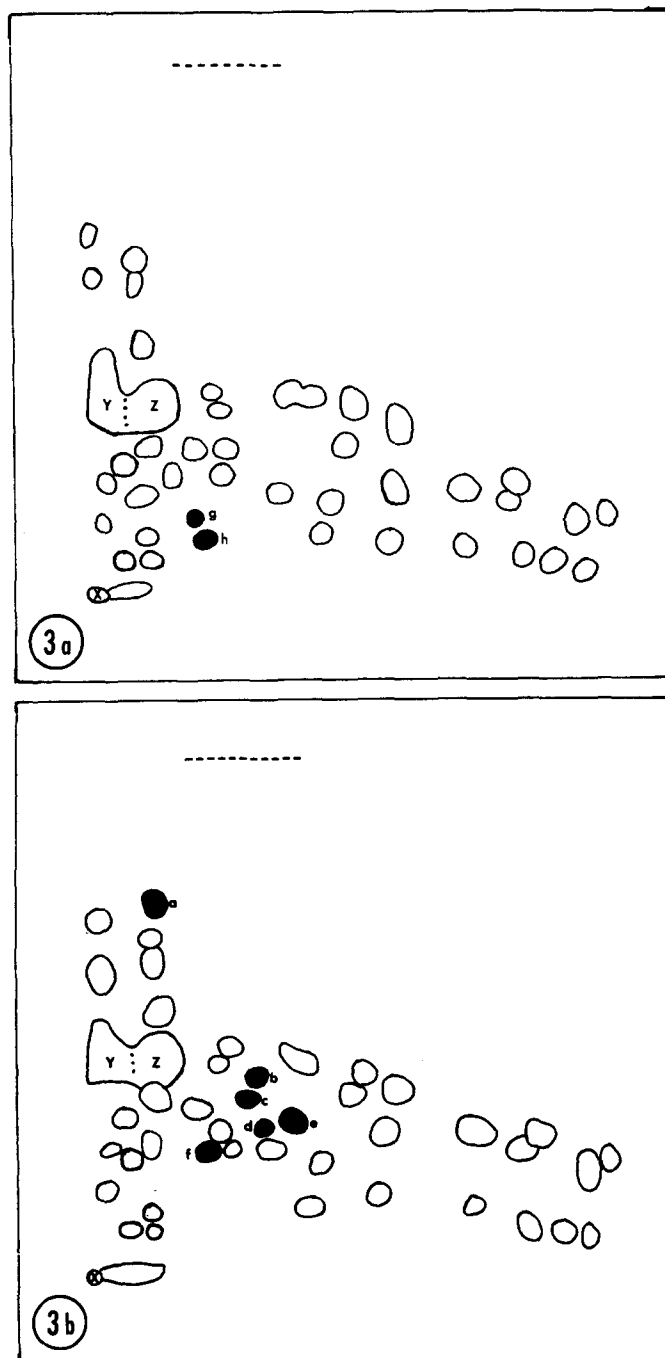


FIGURE 3 Tryptic peptides of tubulin prepared from NES (Fig. 3 *a*) and from WS (Fig. 3 *b*) on silica gel G thin-layer plates as described in the text. Regions Y and Z are groups of peptides which are resolved by less than one spot diameter. Peptides common to both NES and WS tubulin are shown as open circles. Peptides a-e were found exclusively in WS tubulin; peptides g and f exclusively in NES tubulin. Figures are tracings based on duplicate determinations.

specific activity of this protein rose considerably in the interval between 90 min and 24 hr (Fig. 4 *b*). The delayed appearance of labeled protein in NES is believed to be due to transport of this protein from its site of synthesis, presumably in the nerve cell body, to the nerve terminal (11). The present

experiments suggest that a major component of the radioactive soluble protein transferred to the nerve terminal is tubulin. To evaluate this further, protein from NES and WS was purified by the vinblastine procedure at a number of times after intercerebral injection of radioactive leucine.

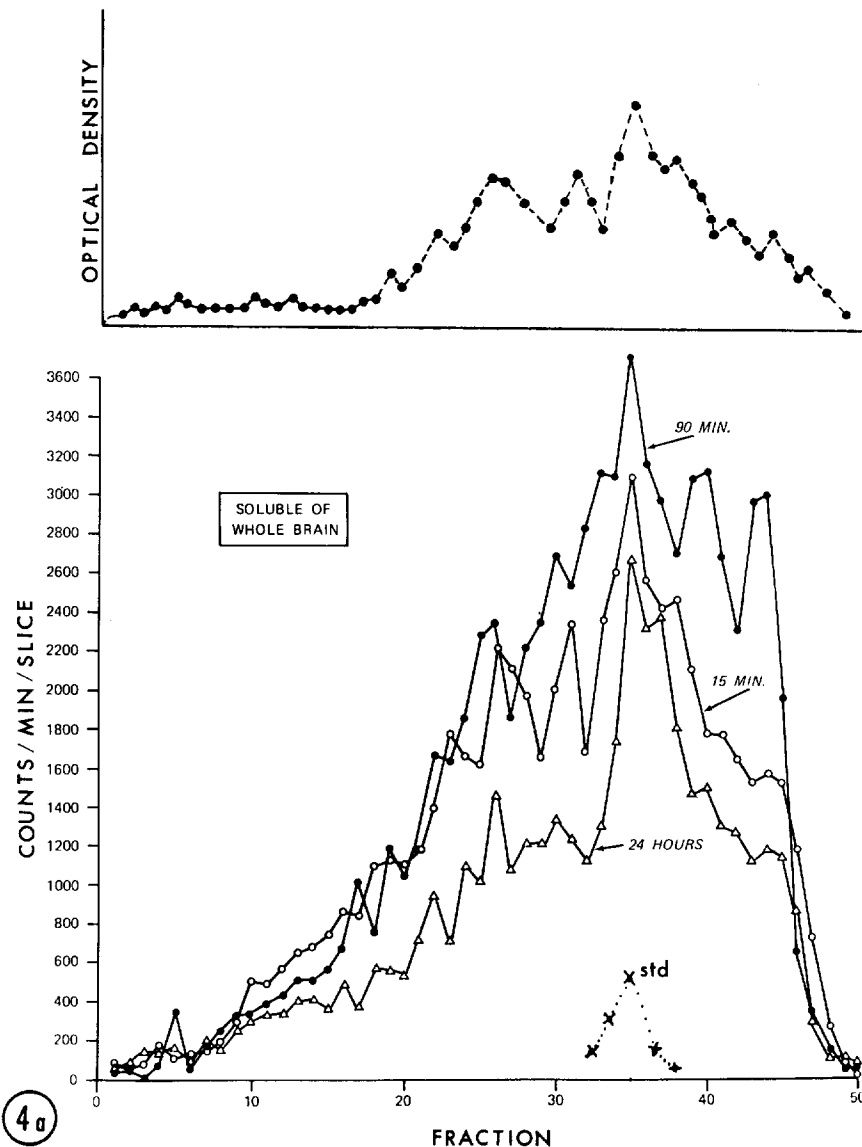
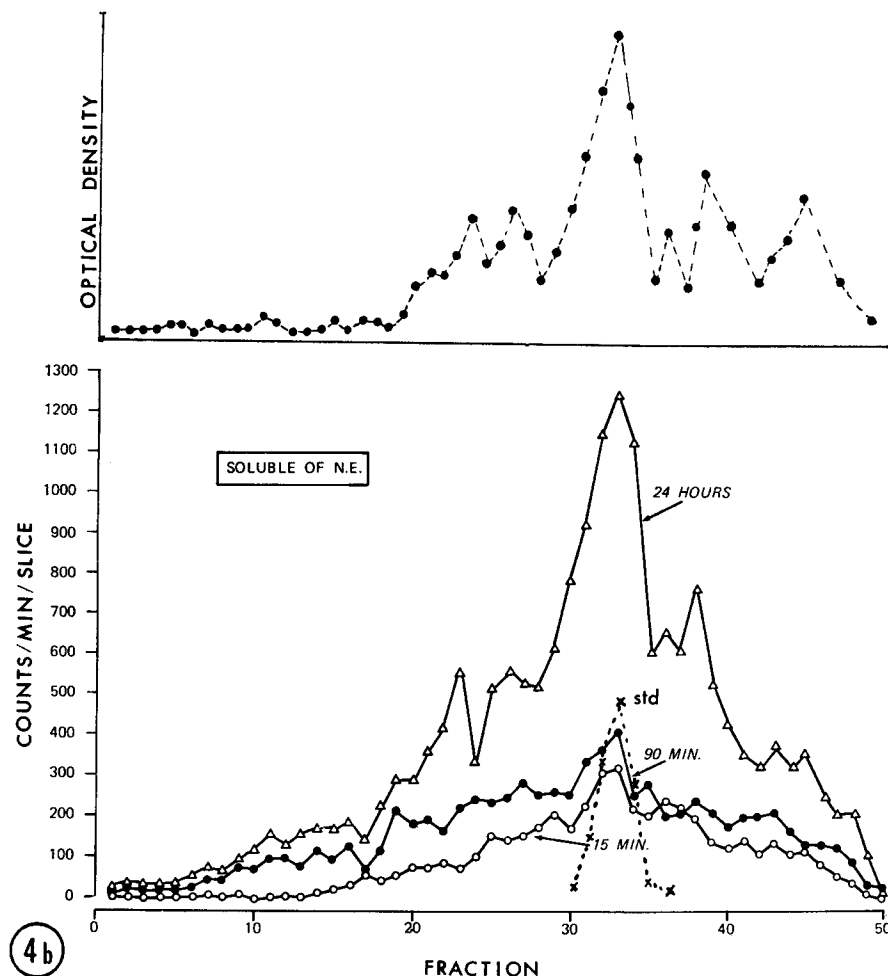


FIGURE 4 Polyacrylamide gel electrophoresis (Materials and Methods, system *a*) of total proteins of WS and NES fractions labeled with tritiated leucine. Groups of six 5-day-old mice were each injected intracerebrally with 10 μ Ci of L-leucine, 4,5- 3 H and sacrificed 15 or 90 min or 24 hr later. WS (*a*) and NES (*b*) were reduced, alkylated, and 0.25 mg protein was electrophoresed along with 50 μ g of tubulin- 14 C. All the counts were multiplied by four so that the data are expressed as if 1 mg of protein was applied to acid gel. The tubulin standard is shown beneath each trace as a dotted line. Dashed lines represent density traces on parallel gels. Fast green stain.



See Fig. 4 a for legend.

Whereas the specific activity of vinblastine-purified protein from WS declined markedly between 90 min after intracerebral injection and 7 days, the specific activity of this protein in NES rose strikingly (Table II) and the ratio of the specific activity of the vinblastine-purified protein in NES over that from WS continued to rise over the 1 wk period in which the study was performed. This indicates the transport of tubulin from a perikaryal site of synthesis to the nerve terminal, and makes unlikely the possibility that NES tubulin is the result of WS contamination.

Other possibilities to explain these results could include synthesis of tubulin at nerve endings. However, the striking lag between administration of radioactive leucine and appearance of labeled tubulin in NES, coupled with the disappearance

of free radioactive leucine within 1 hr of its intracerebral injection (11), argue strongly against this.

DISCUSSION

In a previous report we have shown that nerve ending fractions bind colchicine and that a significant proportion of NES protein is precipitable by vinblastine and is composed of subunits which are monomeric after reduction and alkylation with a molecular weight of approximately 60,000 daltons (9). Although the whole nerve ending fraction bound colchicine, NES had no detectable colchicine binding activity (9). We attributed the loss of colchicine binding activity to the prolonged isolation procedure in the absence of magnesium and the marked dilution of NES during water lysis of the nerve ending fraction;

TABLE II
Specific Activity of Tubulin from NES and WS
Fractions, Purified by Vinblastine
Precipitation

Time after injection	cpm mg protein		
	WS	NES	NES/WS
90 min	175,000	17,000	0.10
1 day	114,000	38,000	0.33
3 days	54,000	49,000	0.91
7 days	27,000	21,000	0.78

NES and WS were isolated at the indicated times after intracerebral injection of groups of eight 5-day-old mice with 10 μ Ci of tritiated leucine into each mouse; and tubulin was purified by vinblastine precipitation, solubilized in buffer, and reprecipitated with cold 5% trichloroacetic acid. Radioactivity and protein were then determined.

but it remained possible that the vinblastine-precipitable protein of NES was not tubulin. The present experiments demonstrate clearly that the electrophoretic migration, amino acid composition, and peptide map of the vinblastine-purified protein from NES is similar to that of tubulin prepared from whole mouse brain. It, therefore, seems quite certain that tubulin is a prominent constituent of the nerve ending fraction.

The present experiments are in accord with the observation that tubulin is slowly transported from the nerve cell body into the nerve ending. Previous studies on axoplasmic transport have demonstrated that intraocularly injected colchicine- 3 H is transported along the optic nerve, presumably bound to tubulin, with a velocity in the range of the slow component of axoplasmic flow (17). Other studies have shown that a substantial portion of the radioactively labeled protein transported in nerve with the slow component of axoplasmic flow is similar to tubulin (18, 19). Although it is clear that at least the great majority of the tubulin of NES is transported there slowly, some labeled tubulin is already detected in NES within 15 min after injection of radioactive leucine. We have previously suggested that some tubulin of NES is transported rapidly to the nerve ending (24), and evidence suggesting rapid transport of some tubulin in the axon has been presented by others (19). The possibility that a component of tubulin is transported rapidly in the axon, and the state of molecular organization of the tubulin which is transported, require further study. Likewise, the

relationship of transport of tubulin in the axon to the effects of vinblastine and colchicine on slow and rapid axoplasmic transport (20-23) remains to be determined.

The small differences in the peptide maps of tubulin from NES and WS may be due to contamination due to variation in purification, but they may be due to small differences in the tubulin itself. It has been demonstrated (25) that proteins may be modified by the addition of glucosamine and other sugars at nerve endings. Other possible changes which would change peptide mapping characteristics include phosphorylation (26), dephosphorylation, and covalent linkage of nucleotide. Recent unpublished results from this labora-

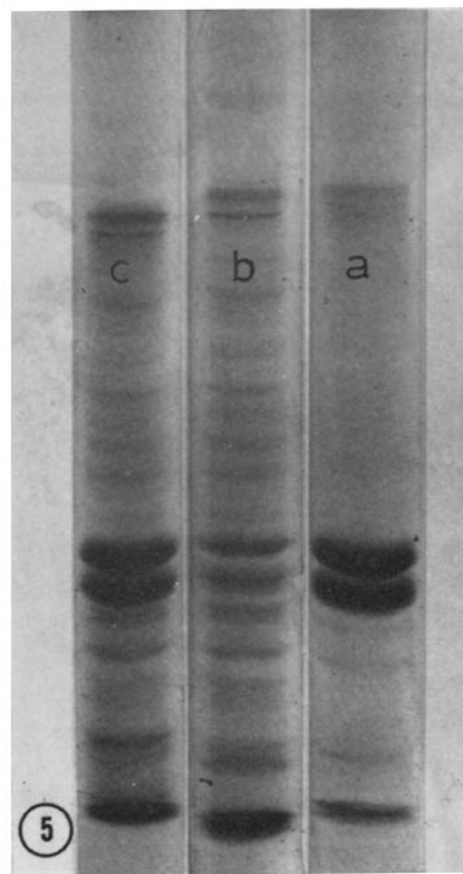


FIGURE 5 Polyacrylamide gel electrophoresis (8 M urea-SDS, Materials and Methods, system c) of tubulin from WS (a), the insoluble proteins remaining after 2X water lysis and high speed centrifugation of the nerve ending fraction (b), and a mixture of the two samples (c).

tory show the presence of hexosamines and the absence of sialic acid in purified tubulin preparations and the incorporation of radioactivity into tubulin at nerve endings after intracerebral injection of glucosamine-¹⁴C. Modifications of this sort might be preliminary to the incorporation of tubulin into membranes, to secretion from the nerve ending, or simply preparatory to degradation.

In previous studies we have demonstrated that the particulate component of isolated nerve ending fractions binds colchicine (9). Electrophoretic study of the proteins of this fraction on SDS-urea gels reveals a large number of bands including two prominent bands which migrate with a velocity identical to that of purified brain tubulin (Fig. 5). This observation strengthens the hypothesis that tubulin may be associated in some manner with membranes at the nerve ending. However, similarity in amino acid compositions and peptide maps must be demonstrated before the presence of tubulin in the particulate fraction can be firmly established.

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